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Published in:
Xenotransplantation

DOI:
10.1111/xen.12403

Publication date:
2018

Document Version
Peer reviewed version

Xenotransplantation

COMMENTARY

Is it currently possible to evaluate the risk posed by PERVs for clinical xenotransplantation?

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Keywords

Porcine endogenous retroviruses, virus safety, solid organ xenotransplantation, islet cell xenotransplantation

The risk of transmission of porcine microorganisms is, in addition to the immunological rejection and the physiological incompatibilities, a major hurdle to the clinical use of pig cells, tissues and organs for the treatment of organ failure in humans, to overcome the medical need caused by the increasing lack of human donors. Whereas most of the porcine microorganisms may be eliminated by early weaning, colostrum deviation, vaccination, antiviral drugs, animal isolation, Caesarean delivery of newborns, and embryo transfer, porcine endogenous retroviruses (PERVs) cannot be eliminated this way because they are integrated in the genome of all pigs [1]. Only a few years before evidence was published that PERV is able to infect human cells [2], two other retroviruses, simian immunodeficiency virus of chimpanzees (SIVcpz) and simian immunodeficiency virus from sooty mangabeys (SIVsm), now called human immunodeficiency viruses 1 and 2 (HIV-1 and 2), invaded the human population causing the fatal acquired immunodeficiency syndrome (AIDS) [3, 4]. Although HIV and PERVs are not very closely related, the fact that PERV is a retrovirus makes it so difficult to evaluate its risk [5]. Also, although most retroviruses are immunosuppressive in the infected host, the absence of an animal model makes it difficult to show this for PERV [6].

In recent years several strategies have been exploited to evaluate the risk posed by PERV, such as (i) infection experiments *in vitro,* (ii) infection experiments *in vivo* in small laboratory animals and in nonhuman primates (NHPs) with and without immunosuppression, (iii) preclinical trials in NHPs transplanting pig cells and organs with and without immunosuppression, and (iv) clinical trials mostly using encapsulated pig islet cells without immunosuppression. Despite these substantial efforts, these studies do not allow to make unequivocal conclusions whether PERVs pose a risk in the case of treatment of humans with porcine cells, tissues or solid organs, as will be discussed in the sections below. Unfortunately, there are no alternative approaches to test this in an experimental setting: essentially clinical trials are needed to answer this question.

1. Infection experiments *in vitro*

There are three types of PERV, PERV-A and PERV-B which are present in the genome of all cells and which infect human cells (human-tropic viruses), and PERV-C which is present in most, but not all pigs and infects only pig cells (ecotropic virus) (for review see [1]). This means that PERV-A and PERV-B are able to infect different human cells and cell lines *in vitro*, in cell culture [2]. Recombinant viruses between PERV-A and PERV-C (PERV-A/C), able to infect human cells and characterized by a high replication rate, been have described [1]. Some human cell lines such as the 293 pig embryonic kidney cell line are highly susceptible, and after repeated passages of PERV through these human cells, the virus showed a higher replication rate and genetic changes in its long-terminal repeat (LTR). These viruses were called “human cell-adapted PERVs” [7]. The lack of the restriction factor apolipoprotein B-editing catalytic polypeptide-like subunit (APOBEC) and transformation by DNA viruses are thought to be the reason for the high susceptibility of 293 cells. However, primary cells including porcine aortic endothelial cells (PAEC) and peripheral blood mononuclear cells (PBMCs) have also been infected [8, 9]. PBMCs can more effectively be infected with human cell-adapted PERVs, however it remains unclear whether the virus infection is productive, e.g., whether the virus infects cells and produces excess progeny [10]. In the case of PAEC, a productive infection including mRNA production and particle release have been demonstrated [8]. Güell et al. [11] described the infection of human umbilical vein endothelial cells (HUVECs) with PERV, and the presence of proviral DNA in the infected cells. However it was not clear how the HUVECs had been derived and whether the infection was productive. Furthermore, all infection experiments, including the experiment with HUVECs, have been performed using PK15 cells or heavily infected 293 cells as virus source, both releasing high amounts of virus. In contrast, most primary pig cells, for example pig PBMCs, show only a low PERV expression at the RNA level and no virus release [12]. Only after mitogenic stimulation of some pig PBMCs, virus particles were released that were able to infect human

293 cells [12, 13]. Therefore, these *in vitro* studies have only a limited relevance for the evaluation whether PERVs pose a risk for xenotransplantation (Table 1).

2. Infection experiments *in vivo*

PERV-A and PERV-B infect not only human cells *in vitro*, but also cells of other species (polytropic viruses), with some exceptions such as mouse cells [1, 14-18]. Based on these results numerous attempts have been undertaken to establish a small laboratory animal model of PERV infection (Table 1). However, injection of PERV preparations into mice, rats, guinea pigs, and minks, with or without immunosuppression, failed to infect these animals [15-19]. Cells from NHPs could also be infected *in vitro*, however in most cases this did not result in a productive infection [17, 19]. In some cases, e.g., chimpanzee cells, only human cell-adapted PERVs were able to show infection [20]. When three NHP species, namely baboons, rhesus monkeys and pig-tailed monkeys, were inoculated with human cell-adapted PERV-A/C, and the animals were treated daily with three different immunosuppressive drugs (cyclosporine, everolimus (RAD), and methylprednisolone), no PERV infection was observed during a follow-up of more than 300 days [17, 21].

Inoculation of rats with PERV or PERV-producing cells [22], or pig islet cells [16], as well as treatment of minks [18] or guinea pigs [22] with PERV did not result in infection. Only in guinea pigs a transient infection was observed [23]. Mice could not be infected [15, 24], because mice lack a PERV receptor [25]. Noteworthy, early reports on PERV infection of SCID mice [26, 27] and athymic mice [28] proved to represent an artifact based on pseudotyping with endogenous murine retroviruses [29, 30]. Mice transgenic for the human PERV receptor huPAR-2 have been generated, and it was reported that they could be infected

with PERV *in vivo* [31]. Although this is the only known *in vivo* model of PERV infection, no follow-up studies on pathogenic effects of the virus were published.

In rhesus and cynomolgus macaques, and baboons, the main virus receptor PAR-1 was found to be genetically deficient by a mutation at the same position as reported in mice, which is one explanation for the inefficient infection [32]. The receptor in African green monkeys does not have this mutation, but nevertheless the replication is quite low [32].

To summarize, all small laboratory animal and NHP model systems are not suitable to evaluate the risk posed by PERVs or to study PERV pathogenesis (Table 1).

3. Preclinical trials in NHPs

In recent years a number of pig-to-NHP preclinical xenotransplantation studies have been performed regarding hearts, kidneys, islet cells, or studies performing perfusion of pig liver, under immunosuppression (for review see [1]). In all these studies, and also in more recent transplantations not listed in [1], i.e., studies on islet cell transplantation in marmosets [33] and cynomolgus monkeys [34], no PERV transmission was observed. However, since the PERV receptor in NHPs is not functional, these results cannot be used to evaluate the safety of xenotransplantation using pig cells and organs (Table 1). Hence, it does not make sense to include the monitoring for PERV transmission in pivotal nonclinical trials before phase transition to clinical development. Interestingly, some regulatory agencies require such studies, which are elaborate and time consuming, and essentially not informative.

4. Clinical trials

In the past, more than 200 humans have received a xenotransplantation product comprising pig cells or tissues including *ex vivo* perfusion of pig organs or pig-cell based bioreactors ([35, 36], reviewed in [1] and [37]. No evidence for virus transmission was obtained using sensitive PCR-based methods and immunological assays for the detection of antiviral antibodies. Neither antibodies against PERV as an indirect sign of infection, nor provirus integration in PBMCs of the patients was observed.

During the last years further clinical trials have been performed, including the first prospective clinical trials under proper regulatory oversight using encapsulated pig islet cells to treat type one-diabetes in New Zealand [38] and Argentina [39]. Although the clinical efficacy in these trials was limited, no PERV transmission has been observed [40, 41].

In all of these porcine islet clinical trials no immunosuppression was given and the islet cells were transplanted encapsulated in biopolymers, a procedure which protects from host´s humoral and cellular immune system (immunoglobulins and immune cells), but also which prevents release of PERVs (Table 1). After some pioneering explorations more than 40 years ago, transplantation of a large vascularized organ accompanied by an effective pharmaceutical immunosuppression has still not been performed.

5. Perspectives

Although human cells can be infected with PERVs under specific and somewhat artificial conditions, i.e., co-culture of human cells with porcine cell lines that do not resemble primary pig cells regarding PERV expression and virus production, or co-culture of porcine cells which human target cell lines that do not resemble primary human cells, no PERV transmission has been observed in the first clinical trials. Also, upon inoculations of PERV

particles or PERV-producing cells into small laboratory animals or NHPs, no PERV transmission has been observed. In addition, no PERV transmission was observed in preclinical trials transplanting encapsulated pig islets in diabetic NHPs or transplanting kidneys or hearts into immunosuppressed NHPs. Noteworthy, the trials and infection experiments in NHPs are limited by the lack of a functional PERV receptor in NHPs. Trials in humans used mainly encapsulated pig islet cells. Encapsulation prevents immune rejection, but could also prevent the release of PERV and other pathogens. *Ex vivo* perfusion of pig liver and spleen by human blood, pig skin transplantations, and injection of pig neuronal cells into the immunoprotected human brain, have also been performed [35, 36]; but till now transplantations of vascularized pig organs under chronic immunosuppression have not been performed. At present there are no additional experimental approaches available to evaluate whether PERVs pose a risk.

During the last years, first reports have been published that PERVs in the genome can be inactivated by CRISPR/Cas9-mediated gene editing tools [42], and also that this procedure allows the generation of live pigs with all PERVs being inactivated [43]. Although the functionality has been shown in *in vitro* cell culture, with inherent low translation value to the pig-to-human clinical situation as outlined above, it needs to be shown in an *in vivo* situation that the inactivation of PERVs in the pig donor makes sense, also in relation to the off-target effects of the gene editing procedure [44].

This aside, the possibility of gene editing resulting in inactivated PERVs raised the question whether conventional pigs can still be used for xenotransplantation, or whether only CRISPR/Cas9-inactivated pigs have to be used as source animals for future xenotransplantations [11, 44-46]. PERV proviruses inactivated by CRISPR/Cas9 cannot be restored by recombination, since in all proviruses the gene coding for the important reverse transcriptase is destroyed. Recombination or co-packaging between PERVs and human

endogenous retroviruses (HERVs) have not been reported [47]. Furthermore, off-target effects by CRISPR/Cas9 may happen, but they will be detected when analyzing the health of the animals, and animals with defects will be eliminated.

Therefore, two options for the first solid organ xenotransplantations could be foreseen. First, the use of organs from conventional, non-CRISPR/Cas9-treated animals in well-controlled trials, e.g., using pigs with absence of PERV-C, low copy number and low expression of PERV-A and PERV-B. Monitoring of the xenotransplant recipient would be as proposed by regulatory agencies [48] using highly sensitive PCR-based and immunological methods. Alternatively, pigs with CRISPR/Cas9-inactivated PERVs could be used. The monitoring might in first instance be similar as mentioned above, considering that the sense of the gene editing can not be demonstrated in *in vivo* animal models [44]. Additional strategies to prevent PERV transmission have been considered such as a vaccine based on neutralizing antibodies [49-52] and antiretroviral drugs (for review see [53]), which may be used should a positive detection of PERV occur. With this in mind, it seems feasible to go ahead with conventional animals as has been done in many trials before.

CONFLICT OF INTEREST

H-J S is director at SchuBiomed Consultancy, and provides consultancy in the biomedical sector worldwide. JD and LS have no conflicts of interest.

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Table 1 Evaluation of the results of different PERV transmission experiments

Setting, method	Outcome PERV transmission	Possible reason why negative	Possible reason why positive	Outlook, conclusion
Clinical transplantation of pig islet and other cells to humans, ex vivo perfusion	No transmission*	Encapsulated cells, low number, no immunosuppression		No relevance for solid organ transplantation into humans
Preclinical transplantation of different pig organs into non-human primates	No transmission	Absence of functional PERV receptor		No relevance for solid organ transplantation into humans
Infection experiments in vivo in small animals and non-human primates (NHP) with and without pharmaceutical immunosuppression	No transmission**	Absence of PERV receptor, or absence of functional PERV receptor, or low PERV receptor density		No relevance for solid organ transplantation into humans
Infection experiments in vitro	Infection of human cells and cells from other species		Use of high virus load for infection, target cells susceptible due to lack of restriction factors, use of human cell adapted virus	Only limited relevance for transplantation into humans, innate and adaptive immune system not present

* In some patients microchimerism was detected, e.g., the presence of pig cells, but no infection [35].

**Reports showing that SCID mice were infected with PERV [26-28] were the result of an artefact based on pseudotyping between PERV and endogenous murine retroviruses [29, 30].